Endothelial nitric oxide synthase gene intron4 VNTR polymorphism in patients with chronic kidney disease

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Nitric oxide production is reduced in renal disease, partially due to decreased endothelial nitric oxide production. Evidence indicates that nitric oxide deficiency contributes to cardiovascular events and progression of kidney damage. A polymorphism in intron 4 of the endothelial constitutive nitric oxide synthase (ecNOS) gene is a candidate gene in cardiovascular and renal diseases. We investigated a potential involvement of this polymorphism in chronic renal failure. A case–control study involved 78 children with chronic kidney disease (CKD) and 30 healthy controls. All participants were genotyped for the ecNOS4 polymorphism by the polymerase chain reaction (PCR). Dialyzed (maintenance hemodialysis) and conservative treatment children had significantly higher frequency of the aa genotype and ecNOS4a allele ($P<0.05$) compared with controls. The combined genotype aa + ab vs. bb comparison validated that a allele is a high-risk allele for end-stage renal disease (ESRD) ($P<0.05$). Serum nitric oxide level was found to be lower in carriers of the ecNOS 4a allele than in noncarriers (100.29 ± 27.32 vs. 152.73 ± 60.39 μmol/l, $P=0.04$). Interestingly, 85.95% of the ecNOS 4a allele ESRD patients were found hypertensive in comparison to the 60.67% patients of non noncarriers (bb genotype) ($P=0.04$). Also, 35.90% of the ecNOS 4a allele ESRD patients were found to have cardiovascular disease in comparison to the 5.13% patients of noncarriers (bb genotype) ($P=0.01$). On multiple linear regression analysis, a allele was independently associated with hypertension ($P=0.03$). There was a significantly higher frequency of the ecNOS4a allele carriers among CKD children, both on MHD and conservative treatment than in controls. This suggests that the ecNOS gene polymorphism may be associated with an increased risk of chronic renal failure. ***Blood Coagul Fibrinolysis*** **22:487–492 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Endothelial function is closely associated with blood pressure and hypertension, and several studies found endothelial dysfunction in normotensive siblings of hypertensive individuals. Endothelial nitric oxide synthase (eNOS), an enzyme that generates nitric oxide, is a major determinant of endothelial function, suggesting that polymorphisms in the eNOS gene may influence the likelihood of blood pressure progression or incident hypertension in an individual person. Nitric oxide is a potent regulator of intrarenal hemodynamics [1]. At the release site it mediates local vasodilatation, antagonizes platelet aggregation and inhibits vascular smooth muscle cell proliferation [2]. In the kidney, nitric oxide dilates renal blood vessels and modulates renin secretion [3]. An impairment of nitric oxide production causes abnormalities in vascular function in many diseases including human hypertension and renal disease [4,5].

The endothelial constitutive nitric oxide synthase (ecNOS), which produces nitric oxide from L-arginine, is encoded by a gene located on chromosome 7q35-36, expressed in endothelium [6]. There are two alleles identified in intron 4 of the ecNOS gene. The larger allele, 4b, consists of five tandem 27-bp repeats and the smaller one, 4a, has four repeats [7]. An association of the 4a allele of the ecNOS gene with coronary heart disease and renal disease was reported [4,8,9].

Chronic renal failure is basically a vascular disorder and investigating ecNOS gene polymorphism might shed some light on the pathophysiology of renal disease and progression to end-stage renal disease (ESRD).

The study investigated: the relevance of the ecNOS intron 4 polymorphism to the development and progression of chronic renal failure; its relationship with hypertension and cardiovascular complications in chronic kidney disease (CKD) pediatric patients undergoing maintenance hemodialysis (MHD) or conservative treatment.

Patients and methods

Seventy-eight pediatric patients with advanced CKD [stages 4 and 5 based on estimated glomerular filtration rate (eGFR) according to the National Kidney Foundation classification] [10] were included in the study. They were divided into two groups undergoing conservative treatment ($n=32$) or MHD ($n=46$). MHD children were

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selected from the hemodialysis unit of the Center of Pediatric Nephrology and Transplantation (CPNT), whereas children undergoing conservative treatment were selected from the Nephrology pediatric clinic, Children's Hospital, Cairo University. The study was done from April 2009 to December 2009. In conservative treatment patients the causes of renal failure were renal hypoplasia or dysplasia ($n=14$), obstructive uropathies ($n=8$), neurogenic bladder ($n=4$), not known ($n=4$), or metabolic ($n=2$). In MHD, the causes of renal failure were hereditary nephropathies ($n=17$), obstructive uropathies ($n=6$), neurogenic bladder ($n=2$), glomerulopathy ($n=2$), renal hypoplasia or dysplasia ($n=2$), and unknown causes ($n=17$). The inclusion criteria for MHD patients included patients with onset of hemodialysis below 16 years with at least 6 months duration on MHD. They were treated with hemodialysis for 3–4 h three times weekly with a polysulfone membrane using bicarbonate-buffered dialysate. The duration of hemodialysis was 2.75 ± 1.59 years. Thirty-two MHD patients and 16 conservative treatment patients were taking antihypertensive treatment.

All control children ($n=30$) were healthy with no clinical signs of vascular or renal disease and no family history of renal disease. An informed consent for genetic studies was obtained from parents of all participants. The protocol of the study was read and approved by the Ethics Committee of NRC in Egypt.

Diagnostic criteria of vascular disease

We studied the prevalence of vascular disease in children with CKD according to the following criteria [11].

Cardiac disease: the presence of primary dilated cardiomyopathy previously diagnosed clinically and by echocardiography.

Cerebral vascular disease: Cerebral vascular disease was suspected on clinical grounds, that is, rapidly developing signs of focal disturbance of cerebral function such as hemiparesis and hemisensory impairment. The diagnosis was confirmed by computed tomography or magnetic resonance imaging. Brain hemorrhage and subarachnoid hemorrhage were excluded.

A patient was considered to have a vascular disease when at least one of these two defined vascular disease was present.

Three millilitres of venous blood sample was collected in EDTA vials for the extraction of genomic DNA. The following parameters were measured: creatinine, urea, electrolytes, total cholesterol, HDL cholesterol and triglyceride by routine methods.

Quantitative determination of nitric oxide concentration in serum

It is done by using the Griss reaction after ultrafiltration via the immunosorbent assay (R&D system Inc., Minneapolis, Minnesota, USA) [12].

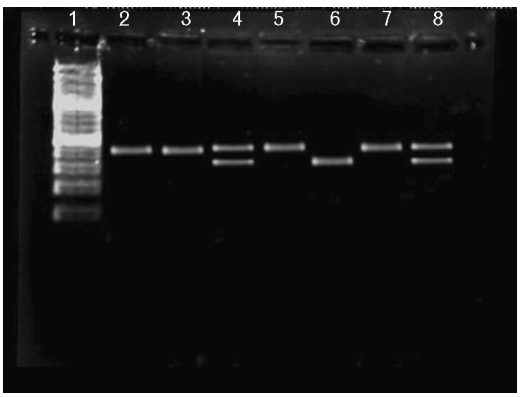
Determination of ecNOS genotype

DNA was extracted from peripheral blood using a QIAamp Blood miniprep extraction Kit (QIAGEN, Germany) and was stored at 4°C until analysis. eNOS genotypes were determined by the polymerase chain reaction (PCR). Briefly, the oligonucleotide primers (the forward primer sequence was 5′-AGGCCCTATGG TAGTGCCTTT -3′ which was located at position 5111–5130 base pairs of the genomic sequence of NOS, and the sequence of the reverse primer was 5′-TCTCTTAGTGCTGT GGTCAC-3′ which its position within the genomic sequence of NOS was 5530–5511 bp) that flank the region of the 27-bp direct repeat in eNOS intron 4 were used for DNA amplification [13]. Each reaction mixture was heated to 94°C for 4 min for denaturation and underwent 35 cycles at 94°C for 1 min, annealing at 56°C for 1 min with an extension at 72°C for 2 min, and a final extension at 74°C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis and fragments were visualized by ethidium bromide staining and ultraviolet transillumination (Fig. 1).

Statistical analysis

Statistical package for social science (SPSS, Chicago, Illinois, USA) program version 11.0 was used for analysis of data. Data were summarized as mean ± SD, range or percentage. Data were evaluated between the experimental groups by one-way analysis of variance (ANOVA). Allele and genotypic frequencies for ecNOS alleles were calculated with the gene counting method. Comparison of the categorical data, that is different ecNOS genotypes among patients was done by independent-samples *t*-test when appropriate. Multiple regression analysis was

Fig. 1



Genotyping of NOS polymorphism photographed on a 2% agarose gel electrophoresis resolving the wild-type allele B at 420 bp and the polymorphic allele A at 393 bp. Lanes (2, 3, 5, and 7) show a single band at 420 bp representing the homozygous allele B pattern (NOS*B*/*B*). Lanes (4 and 8) represent the heterozygous pattern for allele A and allele B (NOS*A*/*B*). Lane (6) shows one band at 393 bp representing the homozygous allele A pattern (NOS*A*/*A*). Lane (1) denotes molecular weight ladder marker 100–1000 bp (Sigma, St. Louis, USA).

performed to assess the influence of ecNOS alleles on hypertension and vascular diseases. A P value of less than 0.05 was considered significant.

Results

Clinical and biochemical characteristics of the studied groups were summarized in Table 1.

There were no significant differences between groups with respect to age, sex ratio and total serum cholesterol level. Serum nitric oxide level was significantly higher in the both MHD and conservative treatment groups than the controls and the level was significantly higher in MHD group than conservative treatment group (143.93 ± 35.99, 91.48 ± 28.80, and 51.84 ± 12.39 μmol/l, respectively).

The distribution of genotypes and allele frequencies was compared between patients and controls (Table 2). The genotype frequencies were in agreement with Hardy–Weinberg equilibrium. Dialyzed and conservative treatment patients had significantly higher frequency of the aa genotype and ecNOS4a allele ($P < 0.05$) compared with control children. The multiple logistic regression analysis with correction for age and sex revealed that the frequency of the ecNOS4a allele carriers was significantly higher in dialyzed and conservative treatment patients than in healthy children. There was no significant difference between MHD and conservative treatment groups as regard to aa genotype ($P > 0.05$).

Comparison of clinical and biochemical characteristics of carriers of the ecNOS 4a allele (aa + ab genotypes) and noncarriers (bb genotype) is shown in Table 3. Interestingly, 85.95% of the ecNOS 4a allele ESRD patients were found hypertensive in comparison to the 60.67% patients of noncarriers (bb genotype) ($P = 0.04$) Also, 35.90% of the ecNOS 4a allele ESRD patients were found to have cardiovascular disease in comparison to the 5.13% patients of noncarriers (bb genotype) ($P = 0.01$). There were significant differences between the two subgroups as regard to triglycerides and HDL cholesterol

Table 2 Frequencies of ecNOS intron 4 genotypes in patients and controls

Gene	CT ($n = 32$)	MHD ($n = 46$)	Controls ($n = 30$)
Alleles			
b	43 (67.19%)	61 (66.30%)	47 (78.33%)
a	21 (32.81%)	31 (33.70%)[b]	13 (22.67%)
Genotypes			
bb	16 (50%)	23 (50.00%)	17 (56.67%)
ab	11 (34.38%)	15 (32.61%)	13 (43.33%)
aa	5 (15.62%)[a]	8 (17.39%)[b]	0 (0.00%)

Data were evaluated by the gene counting method. Values are presented as percentage. CT, conservative treatment; MHD, maintenance hemodialysis. [a] $P < 0.05$ vs. control and CT. [b] $P < 0.05$ vs. control and MHD.

(190.00 ± 57.15 vs. 116.25 ± 48.49 mg/dl, $P = 0.02$; and 21.22 ± 7.05 vs. 32.00 ± 15.96 mg/dl, $P = 0.03$, respectively). Serum nitric oxide level was found to be lower in carriers of the ecNOS 4a allele than in noncarriers (100.29 ± 27.32 vs. 152.73 ± 60.39 μmol/l, $P = 0.04$).

To examine the survival bias in evaluating the effect of the ecNOS4 polymorphism on renal disease, we analyzed allele and genotype frequencies in MHD patients shorter than 3 years on dialysis and those dialyzed 3 years or longer (data not shown). There were no statistically significant differences between these two groups ($P =$ NS), suggesting little or no influence of survival bias on the outcome of the study.

We performed a multiple linear regression analysis with backward stepwise selection using all the clinical risk factors for hypertension and the mutant allele of the eNOS 4a gene. This analysis revealed that the most predictive independent risk factors for hypertension were the mutant allele ($\beta = 0.50$, $P = 0.03$), nitric oxide level ($\beta = -0.47$, $P = 0.03$) and triglyceride ($\beta = 0.60$, $P = 0.02$) (Table 4).

A multiple linear regression analysis using a model identical to the one used to test hypertension was used to test vascular disease for the relationship with the mutant allele of the eNOS 4a gene. This analysis suggested that a allele has a dominant effect on the risk of vascular

Table 1 Clinical and biochemical data of the studied groups

	CT ($n = 32$)	MHD ($n = 46$)	Controls ($n = 30$)
Age	9.14 ± 7.59	10.62 ± 3.49	8.7 ± 4.51
Sex (male/female)	15 (46.88%)/17 (53.12%)	25 (54.35%)/21 (45.65%)	20 (66.67%)/10 (33.33%)
SBP (mmHg)	98.66 ± 6.66	$127.13 \pm 18.37^{b}*$	94.55 ± 9.80
DBP (mmHg)	64.66 ± 6.67	$85.15 \pm 13.76^{b}*$	60.59 ± 10.11
Creatinine (mg/dl)	$3.93 \pm 3.75^{a*}$	$6.32 \pm 1.55^{b}*$	0.77 ± 0.34
Predialysis urea (mg/dl)	$51.12 \pm 10.45^{a*}$	$71.56 \pm 20.61^{b}*$	7.80 ± 2.64
Dialysis (years)		2.75 ± 1.59	
Kt/V		1.69 ± 0.42	
Total cholesterol (mg/dl)	164.44 ± 50.10	$193.04 \pm 51.37^{b}*$	160.31 ± 18.74
Triglycerides (mg/dl)	$160.78 \pm 57.33^{a**}$	$147.00 \pm 66.98^{b}**$	65.31 ± 18.35
HDL cholesterol (mg/dl)	$21.35 \pm 1.17^{a*}$	$27.34 \pm 9.88^{b}*$	39.55 ± 7.94
Serum nitric oxide level (μmol/l)	$91.48 \pm 28.80^{a}*$	$143.93 \pm 35.99^{b**c}$	51.84 ± 12.39

Data were evaluated by ANOVA test. Values are presented as mean ± SD or percentage as applicable. CT, conservative treatment; HDL, high-density lipoprotein; Kt/V, adequacy of hemodialysis; MHD, maintenance hemodialysis. [a]$*P < 0.05$ or [a]$**P < 0.01$ vs. controls and CT. [b]$*P < 0.05$ or [b]$**P < 0.01$ vs. control and MHD. [c]$P < 0.01$ vs. CT and MHD.

Table 3 Clinical characteristics of chronic kidney disease patients with different ecNOS intron 4 genotypes

	aa + ab ($n = 39$)	bb ($n = 39$)	*P* value
Age	11.66 ± 4.77	9.33 ± 3.25	NS
SBP (mmHg)	120.25 ± 22.47	114.00 ± 15.95	NS
DBP (mmHg)	79.37 ± 13.40	77.47 ± 13.80	NS
Hypertensive%	85.95%	60.67%	0.04*
Cardiovascular disease%	14 (35.90%)	2 (5.13%)	0.01*
Total cholesterol (mg/dl)	183.00 ± 38.96	180.00 ± 68.67	NS
Triglycerides (mg/dl)	190.00 ± 57.15	116.25 ± 48.49	0.02*
HDL cholesterol (mg/dl)	21.22 ± 7.05	32.00 ± 15.96	0.03*
Creatinine (mg/dl)	5.99 ± 2.06	5.44 ± 2.15	NS
Predialysis urea (mg/dl)	69.88 ± 27.84	67.00 ± 23.01	NS
Serum NO level (μmol/l)	100.29 ± 27.32	152.73 ± 60.39	0.04*

Significance was estimated using independent t-test. Data are mean ± SD. HDL, high-density lipoprotein; NO, nitric oxide; NS, nonsignificant. * *P* is significant if it is less than 0.05.

complications in these children (ß = 0.99, $P = 0.04$) (Table 5).

Discussion

Chronic renal failure is a multifactorial disease with different prevalence and clinical phenotype in different populations. In this study, the frequency of a allele in the ecNOS intron 4 was significantly higher in both dialyzed and conservative treatment patients compared with healthy controls indicating that the ecNOS 4a allele is a risk factor for ESRD in children with CKD. Such an association was observed earlier by others. Asakimori *et al.* [9] found a significantly higher frequency of the ecNOS 4a allele in hemodialysis patients, both nondiabetic and diabetic, and therefore suggested that the polymorphism in intron 4 of the ecNOS gene may have influence on the progression of renal disease. Wang *et al.* [7] in their study of 302 patients with ESRD and 248 healthy controls found a significantly higher frequency of the ecNOS 4a allele in patients with ESRD caused by nondiabetic primary renal diseases. Freedman *et al.* [14] evaluated the role of four NOS gene polymorphisms in ESRD patients and found that a allele of the ecNOS 4 polymorphism in the NOS gene was associated with all-cause ESRD in probands and their siblings compared with healthy subjects.

In our study, there was a significant elevation of serum nitric oxide levels in patients with CKD either on MHD or on conservative treatment compared to the controls and the level was significantly higher in the MHD group than conservative treatment group. Nitric oxide, an extensively studied endothelium relaxing factor, is reported to be a very potent regulator of intrarenal hemodynamics [15,16]. It plays a major role in the regulation of cardiovascular homeostasis both in health and disease. Many studies have published the relation between nitric oxide and renal failure. An impaired response to nitric oxide may contribute to the initiation or maintenance of the increased intraglomerular high pressure state. The impaired response to nitric oxide appeared more in MHD patients as they are more exposed to increased oxidative stress and this may explain the higher level in this group [17].

Table 4 Risk factors affecting hypertension in chronic kidney disease patients based on multiple linear regression analysis

	β	*P* value
Age	0.06	NS
Triglycerides (mg/dl)	0.60	0.02*
Serum NO level (μmol/l)	0.47	0.03*
Mutant allele (a allele)	0.50	0.03*

NO, nitric oxide. * $P < 0.05$ was considered significant.

Table 5 Risk factors affecting cardiovascular disease in chronic kidney disease patients based on multiple linear regression analysis

	β	*P* value
Age	0.07	NS
Hypertension%	0. 60	0.02*
SBP	2.19	0.03*
DBP	2.60	0.02*
Serum NO level (μmol/l)	0.41	0.03*
Mutant allele (a allele)	0.99	0.04*

NO, nitric oxide. * $P < 0.05$ was considered significant.

In the present study, serum nitric oxide level was found to be lower in carriers of the ecNOS 4a allele than in noncarriers. Gururajan *et al.* [18] reported a strong association between a allele of the ecNOS gene and the plasma NO_x (nitrite and nitrate) levels. The mean plasma level of NO_x of the patients who were homozygous for a allele was nearly 20% lower than in the patients with the b allele. Although it is disputable whether the nitric oxide metabolites in the blood are derived entirely from ecNOS in the endothelial cells of the blood vessels, it is a fact that plasma nitric oxide levels are different depending on ecNOS gene polymorphism. They concluded therefore that the ecNOS gene locus might be responsible for variations in the genetic control of plasma NO_x. The molecular mechanism by which ecNOS gene polymorphism acts to affect the occurrence of ESRD is not known, and it is also unclear whether this polymorphism is a causative variant or a marker of another functional variant. However, the fact that the distribution of a allele in the ecNOS intron 4 showing a significantly higher incidence in children with CKD and plasma nitric oxide metabolite levels are reported to be different in depending on ecNOS gene polymorphism, suggests that the ecNOS intron 4 is a useful marker for studying the relationship between nitric oxide and the progression of renal disease.

The patients of the MHD group have been dialyzed for relatively long periods. Therefore, we examined the survival bias. The frequencies of ecNOS intron 4 gene polymorphism in the long dialysis period group did not differ from those in the short period group. It appears therefore, that neither death nor survival is factor in

estimating the role of gene polymorphism in disease progression.

In this study, 85.95% of the ecNOS 4a allele ESRD patients were found hypertensive in comparison to the 60.67% patients of noncarriers (bb genotype) and on correlating the DBP to a allele and other individual variables by multiple linear regression analysis, a allele, nitric oxide level and triglyceride concentration were variables that were independently associated with DBP ($P < 0.05$). A study using mice with disrupted eNOS gene revealed that eNOS function is required for vascular and hemodynamic responses to acetylcholine and that the disruption of the eNOS gene leads to hypertension [19]. Clinical and experimental studies suggest that an alteration in nitric oxide metabolism may be a contributing factor in the pathogenesis of hypertension. Thus, abnormalities in the activity of the enzyme eNOS that synthesizes nitric oxide in endothelial cells may lead to nitric oxide abnormality with severe consequences [20]. Inhibition of eNOS elevates blood pressure in healthy humans [21]. Furthermore, nitric oxide production is diminished in patients with essential hypertension, under basal conditions [22].

Our study revealed that 35.90% of the ecNOS 4a allele ESRD patients were found to have cardiovascular disease in comparison to the 5.13% patients of noncarriers (bb genotype) and that the allele has a dominant effect on the risk of vascular complications in children with CKD by multiple linear regression analysis. Apart from controlling the coronary blood flow, there is now an emerging consensus that nitric oxide generally acts to fine-tune and optimize cardiac pump function [23]. Excessive nitric oxide depresses systolic function by decreasing myocardial contractility and shortening the ejection period [23]. Elevated circulating levels of oxidative products of (NO_x) and myocardial nitric oxide synthetase expression have been seen in patients with heart failure due to contractile dysfunction [24,25]. We had a previous study on the relation between plasma nitric oxide level and left-ventricular (LV) diastolic function and its cause in heart failure patients in the pediatric age group. We found that the plasma NO_x levels are elevated in patients with isolated diastolic heart failure; in addition, in patients with LV systolic failure, the severity of LV diastolic dysfunction determines the amount of nitric oxide production [26]. Many studies reported a correlation of this polymorphism with vascular diseases [27–29].

In summary, a allele of the ecNOS intron 4 gene polymorphism showed a significantly higher frequency in children with CKD, both on MHD and conservative treatment. These results suggest that the ecNOS gene polymorphism can serve as a useful genetic marker for evaluation of susceptibility to chronic renal failure. However, the interactions between this genetic predisposition and environmental factors as well haplotype analysis need more studies.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Tolins JP, Palmer RM, Moncada S, Raij L. Role of endothelium-derived relaxing factor in regulation of renal hemodynamic responses. *Am J Physiol* 1990; **258**:655–662.
2 Schmidt HH, Walter U. NO at work. *Cell* 1994; **78**:919–925.
3 Kone BC, Baylis C. Biosynthesis and homeostatic roles of nitric oxide in the normal kidney. *Am J Physiol* 1997; **272**:F561–F578.
4 Miyamoto Y, Saito Y, Kajiyama N, Yoshimura M, Shimasaki Y, Nakayama M, *et al.* Endothelial nitric oxide synthase gene is positively associated with essential hypertension. *Hypertension* 1998; **32**:3–8.
5 Schmidt RJ, Baylis C. Total nitric oxide production is low in patients with chronic renal disease. *Kidney Int* 2000; **58**:1261–1266.
6 Nadaud S, Bonnardeaux A, Lathrop M, Soubrier F. Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. *Biochem Biophys Res Commun* 1994; **198**:1027–1033.
7 Wang Y, Kikuchi S, Suzuki H, Nagase S, Koyama A. Endothelial nitric oxide synthase gene polymorphism in intron 4 affects the progression of renal failure in nondiabetic renal diseases. *Nephrol Dial Transplant* 1999; **14**:2898–2902.
8 Hibi K, Ishigami T, Tamura K, Mizushima S, Nyui N, Fujita T, *et al.* Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. *Hypertension* 1998; **32**:521–526; ecNOS gene polymorphism in renal failure 2305.
9 Buraczynska M, Ksiazek P, Zaluska W, Nowicka T, Ksiazek A. Endothelial nitric oxide synthase gene intron 4 polymorphism in patients with end-stage renal disease. *Nephrol Dial Transplant* 2004; **19**:2302–2306.
10 National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* 2002; **39(Suppl 1)**: S1–S266.
11 Kimura H, Gejyo F, Suzuki S, Yamaguchi T, Imura T, Miyazaki R, *et al.* Cholesteryl ester transfer protein gene mutation and vascular disease in dialysis patients. *J Am Soc Nephrol* 1999; **10**:294–299.
12 Tsikas D. Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. *Free Radic Res* 2005; **39**:797–815.
13 Salimi S, Firoozrai M, Nourmohammadi I, Shabani M, Mohebbi A. Endothelial nitric oxide synthase gene intron4 VNTR polymorphism in patients with coronary artery disease in Iran. *Indian J Med Res* 2006; **124**:683–688.
14 Freedman BI, Yu H, Anderson PJ, Roh BH, Rich SS, Bowden BW. Genetic analysis of nitric oxide and endothelin in end-stage renal disease. *Nephrol Dial Transplant* 2000; **15**:1794–1800.
15 Kerkeni M, Letaief A, Achour A, Miled A, Trivin F, Maaroufi K. Endothelial nitric oxide synthetase, methylenetetrahydrofolate reductase polymorphisms, and cardiovascular complications in Tunisian patients with nondiabetic renal disease. *Clin Biochem* 2009; **42 (10–11)**:958–964.
16 Möllsten A, Lajer M, Jorsal A, Tarnow L. The endothelial nitric oxide synthase gene and risk of diabetic nephropathy and development of cardiovascular disease in type 1 diabetes. *Mol Genet Metab* 2009; **97**:80–84; Epub 2009 Feb 3.
17 Nguyen-Khoa T, Massy ZA, de Bandt JP, Kebede M, Salama L, Lambrey G, *et al.* Oxidative stress and hemodialysis: role of inflammation and duration of dialysis treatment. *Nephrol Dial Transplant* 2001; **16**:335–340.
18 Gururajan P, Gurumurthy P, Victor D, Srinivasa Nageswara Rao G, Sai Babu R, Sarasa Bharati A, Cherian KM. Plasma total nitric oxide and endothelial constitutive nitric oxide synthase (ecNOS) gene polymorphism: a study in a South Indian population. *Biochem Genet* 2010; **10528**:9391–9397.
19 Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 1995; **377**:239–242.
20 Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989; **2**:997–1000.
21 Haynes WG, Noon JP, Walker BR, Webb DJ. Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J Hypertens* 1993; **11**:1375–1380.
22 Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N. Basal nitric oxide synthesis in essential hypertension. *Lancet* 1997; **349**:837–842.
23 Cotton JM, Kearney MT, Shah AM. Nitric oxide and myocardial function in heart failure: friend or foe? *Heart* 2002; **88**:564–566.

24 Balat A, Cekmen M, Yurekli M, Yilmaz K, Sahinoz S. Adrenomodulin and nitrite in children with dilated cardiomyopathy. *Pediatric Cardiol* 2003; **24**:381–385.
25 Fukuchi M, Hussain S, Giaid A. Heterogenous expression and activity of endothelial and inducible nitric oxide synthases in end stage human heart failure. *Circulation* 1998; **19**:132–139.
26 Elshamaa MF, Sharaf EA, Farid YA, Elghoroury EA, Abdelghaffar E. Plasma nitric oxide level in myocardial disorders with left ventricular diastolic dysfunction. *J Clin Basic Cardiol* 2006; **9**:Online 23–26.
27 27Mitsuke Y, Lee JD, Shimizu H, Uzui H, Iwasaki H, Ueda T. Nitric oxide synthase activity in peripheral polymorphonuclear leukocytes in patients with chronic congestive heart failure. *Am J Cardiol* 2001; **87**:183–187.
28 Thibaud D, Philippe R, Ajay M, Emmanuel C, Isabelle M, Hassenfuss G, *et al.* Increased neuronal nitric oxide synthase derived NO production in the failing human heart. *Lancet* 2004; **363**:1365–1367.
29 Wasson S, Govindarajan G, Reddy HK, Flaker G. The role of nitric oxide and vasopressin in refractory right heart failure. *J Cardiovascpharm Ther* 2004; **9**:9–11.